RNA-Seq workflow

Phase 1: developing a workflow and preprocessing raw reads

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`https://github.com/PiscatorX/RNA-Seq-devs`

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Overview

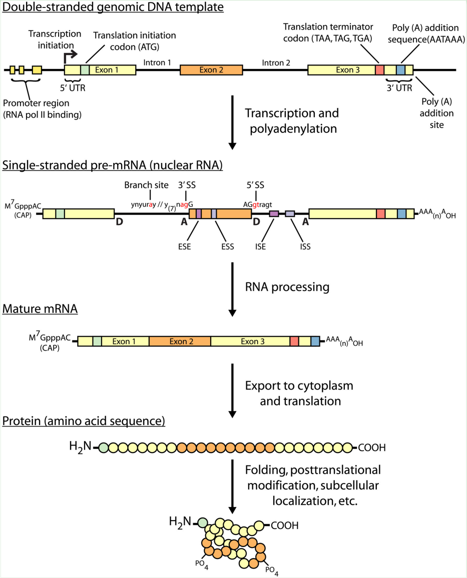
1. overview
2. Background: why RNA?
3. Ecological transcriptomics
4. RNA-seq workflow
5. Reference and *denovo* RNA-seq studies

Why RNA?

Why should I do RNA-seq, I have a genome?

- Insights into functional responses
- Genome = potential gene function
- RNA is better proxy for proteins than genome
- Genomes overemphasise mutations
  - Alternative splicing of isoforms, fusion transcripts
  - RNA interference genes may be silenced
  - Same genome in all cells and tissue types e.g brain cells and liver cells
  - Gene models for the identification of transcripts remains a challenge
- changes in experimental conditions = changes in gene expression
- Expression profile is a fingerprint of cell or tissue type
- Some genes are driven by gene-gene interations e.g epistasis

DNA makes RNA, and RNA makes protein



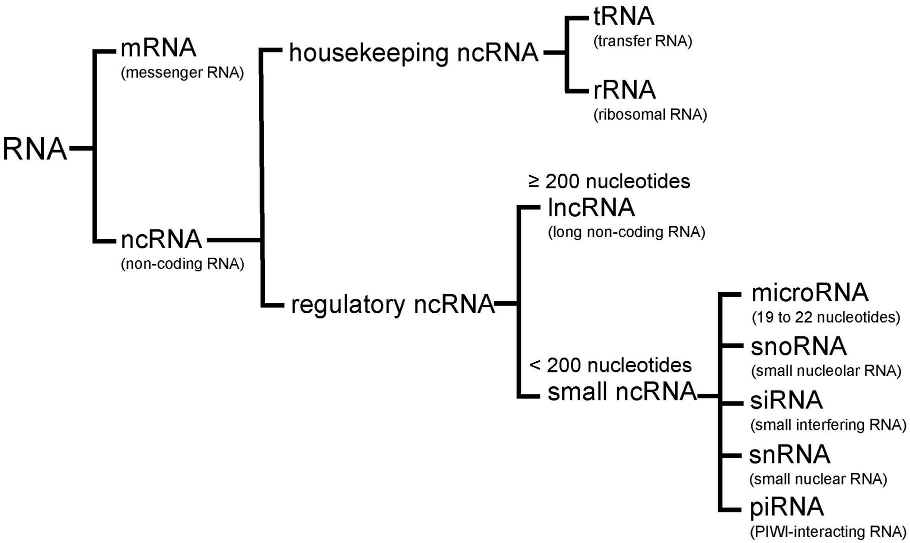
Double-stranded genomic DNA template
Transcription initiation
Translation initiation codon (ATG)
Translation terminator codon (TAA, TAG, TGA)
Poly (A) addition sequence(AATAAA)
Exon 1
Intron 1
Exon 2
Intron 2
Exon 3
5' UTR
3' UTR
Promoter region (RNA pol II binding)
Poly (A) addition site
Transcription and polyadenylation
Single-stranded pre-mRNA (nuclear RNA)
Branch site
3' SS
5' SS
ynyuray // y(7)nagG
AGgtragt
M7GpppAC (CAP)
D
A
D
A
AAA(n)AOH
ESE
ESS
ISE
ISS
RNA processing
Mature mRNA
M7GpppAC (CAP)
Exon 1
Exon 2
Exon 3
AAA(n)AOH
Export to cytoplasm and translation
Protein (amino acid sequence)
H2N
COOH
Folding, posttranslational modification, subcellular localization, etc.
H2N
COOH
PO4
PO4

- We want to study proteins but they are hard to identify
- So we study RNA as a proxy for proteins

Abbreviations:
D=donor splice site;
A=acceptor splice site;
poly (A)=polyadenylation;
UTR= untranslated region;
SS=splice site;
ESE=exonic splicing enhancer;
ESS=exonic splicing silencer;
ISE=intronic splicing enhancer
ISS=intronic splicing silencer

Griffith et al., 2015

There are many RNA types



Ecological transcriptomics

Transcriptome

"The transcriptome is the complete set of transcripts in a cell, and their quantity, for a specific developmental stage or physiological condition"

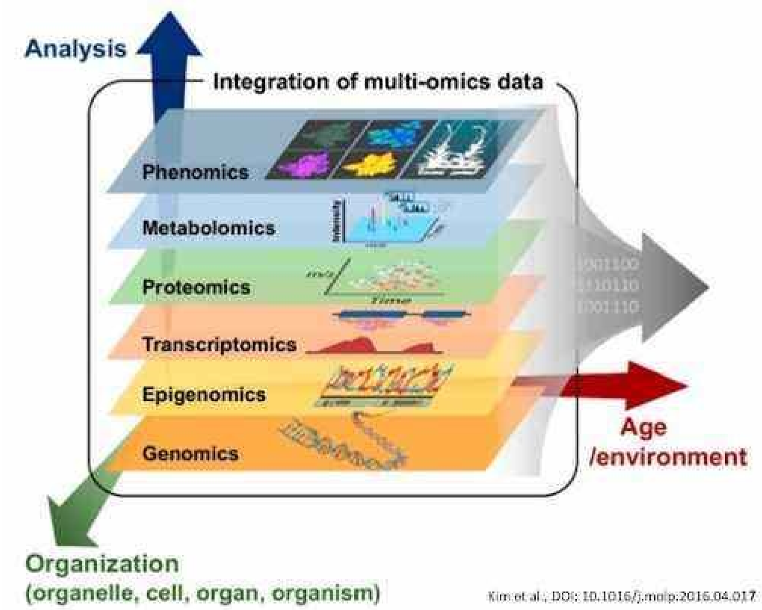
Wang et al., 2009

Ecological transcriptomics?

- Integration of transcriptomic analysis with ecological studies.
- Elucidation the genomic basis of phenotypic variation of individuals in response to environmental changes under natural or laboratory conditions

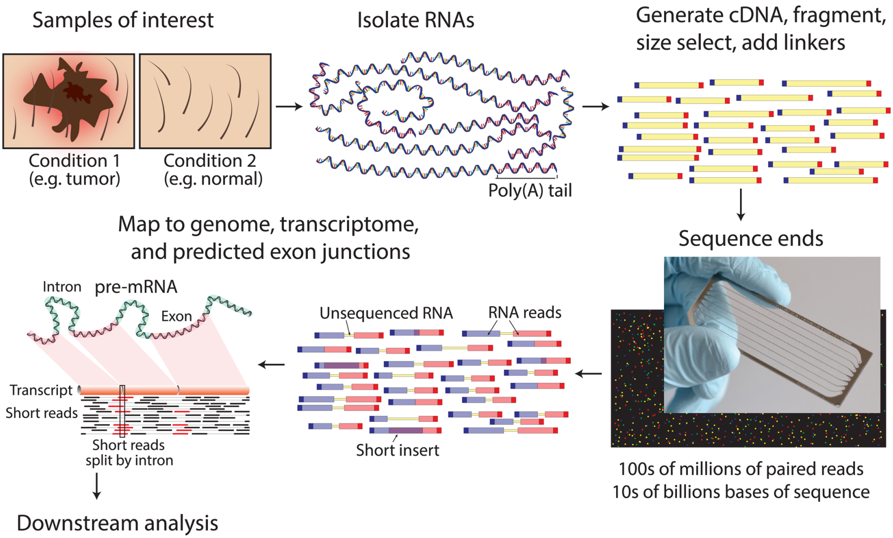
Richards et al., 2009; Alvarez et al., 2015

Omics layers:



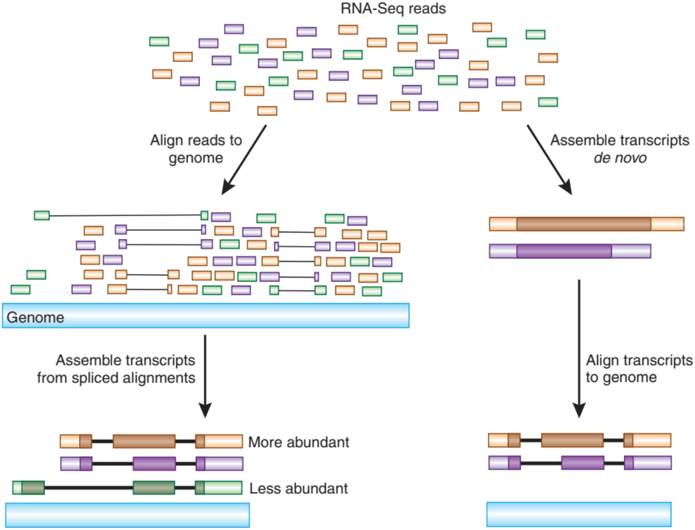
Kim et al., DOI: 10.1016/j.molp.2016.04.017

Overview of RNA-seq workflow



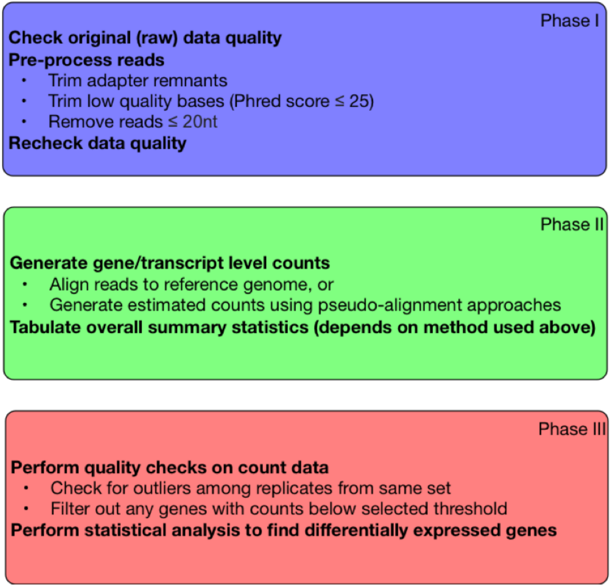
Griffith et al., 2015

Reference and *denovo* RNA-seq studies


RNA-Seq reads
Align reads to genome
Assemble transcripts *de novo*
Genome
Assemble transcripts from spliced alignments
Align transcripts to genome
More abundant
Less abundant

Haas and Zody, 2010

Overview of plan of workflow development



Phase I

Check original (raw) data quality

Pre-process reads

- Trim adapter remnants
- Trim low quality bases (Phred score ≤ 25)
- Remove reads ≤ 20nt

Recheck data quality

Phase II

Generate gene/transcript level counts

- Align reads to reference genome, or
- Generate estimated counts using pseudo-alignment approaches

Tabulate overall summary statistics (depends on method used above)

Phase III

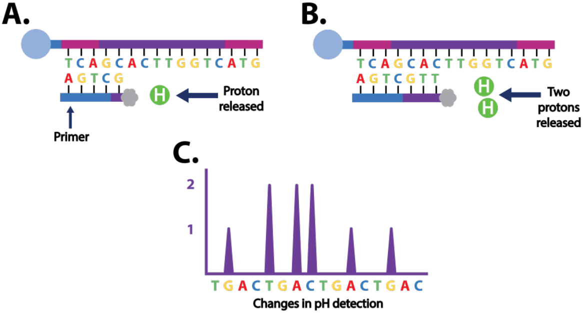
Perform quality checks on count data

- Check for outliers among replicates from same set
- Filter out any genes with counts below selected threshold

Perform statistical analysis to find differentially expressed genes

`https://h3abionet.github.io/H3ABionet-SOPs/RNA-Seq-1-2.html`

Ion torrent (Ion semiconductor sequencing)



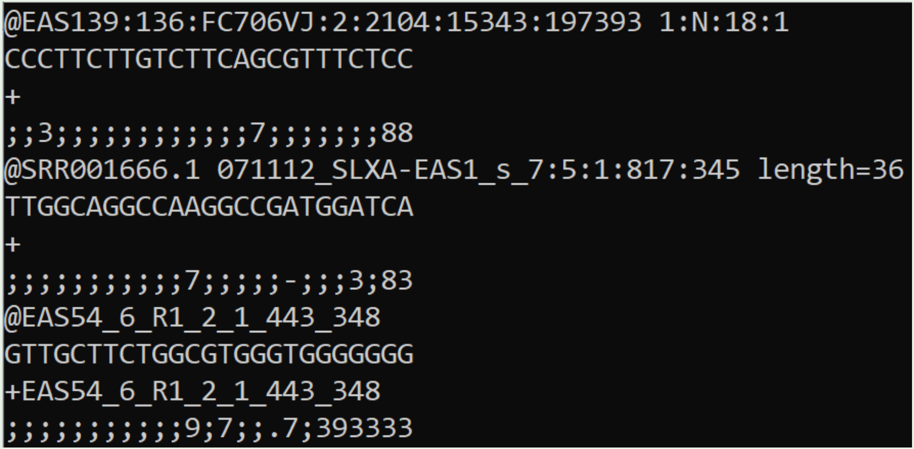
https://apollo-institute.org/ion-torrent-sequencing/

Comparison of specifications of Illumina and Ion Torrent platforms.

Platform	Illumina MiSeq	Ion S5/Ion S5 Plus/Ion S5 Prime
Sequence yield per run	7.5—8.5 Gb on reagents v.2 12.5—15 Gb on reagents v.3	1,2-2 Gb on 520 chip 6-8 Gb on 530 chip
Accuracy	70% > Q30 at 600 cycles, 85% > Q30 at 500 cycles	85% > Q20
Systematic error	substitutions in GGC and GGT context	indels in homopolymer regions
Read length	500 (250 + 250) bp for reagents v.2 600 (300 + 300) bp for reagents v.3	~400 bp for double chip up to ~600 bp for single chip
Run Time	39 h for 500 cycles 56 h for 600 cycles (includes cluster generation, sequencing and base calling)	19,5 h for 400 bp (includes presequencing chip processing, initialization a sequencing)
Paired reads	Yes	No
Insert size	up to 550 bp	400 bp (up to 600 bp)

Step 1.1: Quality check

Example fastq file



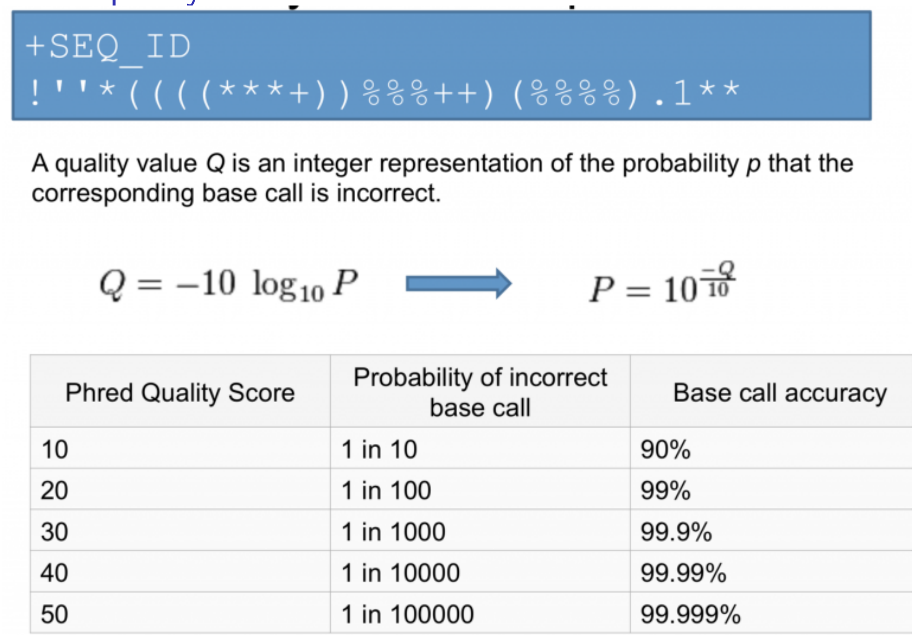
```
@EAS139:136:FC706VJ:2:2104:15343:197393 1:N:18:1
CCCTTCTTGTCTTCAGCGTTTCTCC
+
;;3;;;;;;;;;;;;7;;;;;;;88
@SRR001666.1 071112_SLXA-EAS1_s_7:5:1:817:345 length=36
TTGGCAGGCCAAGGCCGATGGATCA
+
;;;;;;;;;;;7;;;;;-;;;3;83
@EAS54_6_R1_2_1_443_348
GTTGCTTCTGGCGTGGGTGGGGGGG
+EAS54_6_R1_2_1_443_348
;;;;;;;;;;;9;7;;.7;393333
```

Sequence reads: FASTQ Format Specification

- First line is the sequence header which starts with an ‘@‘ (not a ‘>’)
- Everything from the leading ‘@‘ to the first whitespace character is considered the sequence identifier
- Everything after the first space is considered the sequence description
- Second line is the sequence
- Third line starts with ‘+’ and can have the same sequence identifier appended (but usually doesn’t anymore)
- Fourth line are the quality scores

https://en.wikipedia.org/wiki/FASTQ_format
https://learn.gencore.bio.nyu.edu/ngs-file-formats/fastq-format/

Phred quality scores



```
+SEQ_ID
!''*((((***+))%%%++)(%%%%).1**
```

A quality value *Q* is an integer representation of the probability *p* that the corresponding base call is incorrect.

$$Q = -10\ \log_{10} P \longrightarrow P = 10^{\frac{-Q}{10}}$$

Phred Quality Score	Probability of incorrect base call	Base call accuracy
10	1 in 10	90%
20	1 in 100	99%
30	1 in 1000	99.9%
40	1 in 10000	99.99%
50	1 in 100000	99.999%

FASTQC: A quality control tool for high throughput sequence data

https://h3abionet.github.io/H3ABionet-SOPs/RNA-Seq-2-1.html

Before we start: an brief interlude on installing your tools

I highly recommend learning how to use conda
https://conda.io/projects/conda/en/latest/index.html

Running FastQC

```
fastqc seqfile1 seqfile2 .. seqfileN

fastqc [−o output dir] [−−(no)extract] [−f fastq|bam|sam]
        [−c contaminant file] seqfile1 .. seqfileN
```

https://www.bioinformatics.babraham.ac.uk/projects/fastqc/

MultiQC

Aggregate results from bioinformatics analyses across many samples into a single report

`https://multiqc.info/modules/fastqc/`

- Analyses the output files of analysis tools which generate multiple files
- Aggregates into one file
- Quick overview of metrics in one place
- Lots of supported tools `https://multiqc.info/modules/`

Trimmomatic: A flexible read trimming tool for Illumina NGS data

`http://www.usadellab.org/cms/?page=trimmomatic`

1. Trimming
   - Removing low-quality bases or regions at the ends of reads
   - Improving the overall quality of the reads
2. Adapter removal
   - Detecting and removing residual adapter sequences present in the reads
   - Adapter sequences can interfere with downstream alignment/mapping
3. Size selection
   - Filtering reads based on their length to meet specific application requirements
   - Selecting reads within the desired length range

```
trimmomatic SE [-version] [-threads <threads>] \
[-phred33|-phred64] [-trimlog <trimLogFile>] \
[-summary <statsSummaryFile>] \
[-quiet] <inputFile> <outputFile> <trimmer1>...
```